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TITLE: Determination of Catechol Estrogen Adducts by High-
Performance Liquid Chromatography: Establishing
Biomarkers for the Early Detection of Breast Cancer

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13. ABSTRACT (Maximum 200 Words) In order to better understand the role of estrogen metabolism as it relates to breast cancer etiology, a new analytical technique that can measure CE and CE-DNA adducts at low endogenous levels is being developed. This new technique is based on HPLC analysis of fluorescent probes specific for CE and CE-DNA adducts. An extraction procedure employing a solvent mixture of chloroform, dimethylformamide, and acetic acid was developed to extract CE-DNA adducts, CE, and MPEM from rat breast tissue. This extraction procedure involves homogenization of the tissue in 20 mL of the solvent mixture, removal of the solvent, followed by HPLC analysis. Reaction of α,α -dibromomalonamides occurs quickly with catechols, and this malonamide system is being developed to produce fluorescent probes for HPLC analysis. Reaction of N,N'-Bis-anthracen-9-ylmethyl-2,2-dibromo-malonamide with catechol results in ketal formation. The resulting product is highly fluorescent and can be detected at the low femtomole level. The aim of this work is to develop new biomarkers for the early detection of breast cancer.				
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INTRODUCTION:

The metabolism of estrogen to procarcinogenic catechols has been hypothesized as an initiation step in the development of breast cancer [1-4]. Specifically, the over expression of 4-hydroxylase activity has been observed in organs prone to estrogen-induced tumors [5-8]. The relationship between increased production of 4-hydroxyestradiol (4-OHE₂) and 4-hydroxyestrone (4-OHE₁) metabolites with the increase occurrence of estrogen-induced tumors is still not clear. Hypothesis regarding redox cycling [9-13] and oxidation to electrophilic quinones have been examined [3,4]. Since oxidation of catechol estrogens (CE) to catechol estrogen quinones (CE-Q) has been shown to lead to CE-DNA adducts [4], we seek to develop an analytical technique that can measure these adducts at endogenous levels. The purpose of developing this assay is to examine whether CE-DNA adducts are present in breast cancer tissue. This would be a first step in understanding the etiology of breast cancer as it relates to estrogen metabolism, specifically, the role of 4-hydroxylase activity in the increase occurrence of estrogen-induced cancers. The scope of this method development involves the production of fluorescent probes, specific for the catechol moiety, so that low-level detection of these adducts can be accomplished. With the proper development of fluorescent probes, this assay would be very sensitive and selective towards CE-DNA adducts.

BODY:

Work related to Task 1, "Develop an extraction procedure for the isolation of CE-adducts, CE and MPEM from rat mammary tissue", has been completed. CE-DNA adducts, CE, and monophenol estrogen metabolites (MPEM) were added to ca. 0.5 g of rat mammary tissue (Sprague Dawley). The compounds were applied to the tissue in dimethylformamide (DMF) solutions and allowed to stay in contact with the tissue for one hour at room temperature. The tissue was then homogenized in a 50 mL centrifuge tube for 2 minutes using 20 mL of an optimized solvent mixture. The solvent mixture consisted of 55:40:5, chloroform, DMF, and acetic acid, respectively, containing 0.5g-per 100mL ascorbic acid. This solvent mixture has several advantages: 1) by employing greater than 50% chloroform, the mixture is non-flammable and can be used by conventional, metal-based homogenizers; 2) the chloroform-DMF mixture extracts both polar CE-DNA adducts, and the less polar MPEM; 3) the acetic acid, in combination with added ascorbic acid impedes air oxidation of catechol metabolites. Thus, 4-OHE₁-1-N7Gua (CE-DNA adduct), 4-OHE₁ (CE), and 4-methoxyE₁ (MPEM) were placed on the rat tissue in 20μL of DMF containing 100 nmol of the respective compound (see Figure 1 for metabolite structures). The tissue was homogenized for 2 minutes using 20 mL of the solvent mixture. The suspension was then centrifuged, the solvent decanted, and the decanted solvent was removed by reduced pressure evaporation. The residue was then re-dissolved into 100 μL of clean DMF and a 50μL aliquot was injected on a HPLC employing a C-18 reverse phase column. The compounds were eluted using a gradient of 0.5 percent acetic acid and methanol and detected by UV absorbance (280 nm). Using standards for calibration curves, recoveries for all compounds were assayed at 80 to 90%.

Work related to Task 2, "Develop an HPLC analytical procedure, via pre-column fluorescence derivatization, for the femtomolar detection of CE-adducts, CE and MPEM in human breast tissue", has been completed. Development of a fluorescent probe specific for the catechol moiety was conducted by developing structures that can react with the vicinal hydroxy groups selectively (see Figure 2). Since the vicinal hydroxy groups can act as nucleophiles, structures containing activated geminal dihalides have been synthesized and shown to react with catechols forming a spiro, ketal derivative. Several dihalide candidates have been developed (Year 1, Year 2, and Year 3 report) with α,α-dibromomalonamides showing the most promise. Work this past year on Task 2 has involved the synthesis of α,α -dibromomalonamide probes containing anthracene moieties. An amide linkage to the fluorophore was developed in order to overcome problems associated with the ester linkage of the bis-(9-methyleneanthryl)-α,α -dibromomalonate probe (discussed below).

The synthesis of bis-(9-methyleneanthryl) α,α -dibromomalonate was conducted using several methods (Year 3 report); however, the reaction of this probe with catechol proved unsuccessful. Unlike

the diethyl analog (Figure 3), the dianthryl probe produced 9-anthrylmethanol as the sole product in nearly quantitative yield (Figure 4). Several variations of solvent, base, and reaction temperature were tried with no success. Bis-(1-butylpyrene) α,α -dibromomalonate was also synthesized, but reaction with catechol again produced only alcohol side products. One possible difference for the reaction of the ethyl ester compared with esters containing anthracene or pyrene groups is the propensity for both anthracene and pyrene towards bromination. Dibromomalonates have been employed as brominating reagents [14]. An indirect method of attaching the fluorophore to the ester-ketal product was conducted (see Figure 5), but this process required several steps and produced several unknown by-products that complicated HPLC separation.

Since the ester probes led to alcohol products via an unknown decomposition process, we sought to synthesize an amide analog of the same dianthracene probe. The amide linkage is less prone to hydrolysis when compared to esters, and thus a probe based on an amide linkage should react with catechols without decomposition side-products. Figure 6 details the synthetic sequence used to produce N,N'-Bis-anthracen-9-ylmethyl-2,2-dibromo-malonamide. The convergent synthesis uses malonic acid and 9-anthracenemethanol as commercially available starting materials. Bromination of the malonic acid to α,α -dibromomalonic acid, followed by conversion to an acid chloride produces α,α -dibromomalonoyl chloride. When 2.5 equivalents of 9-Anthracen-9-yl-methylamine, produced in three steps from 9-anthracenemethanol, is reacted with α,α -dibromomalonoyl chloride, the fluorescent probe N,N'-Bis-anthracen-9-ylmethyl-2,2-dibromo-malonamide (**1**) is generated in fair yield.

The reaction of (**1**) with the estrogen-DNA adducts 4-OHE1-1-N7Gua and 4-OHE1-1-N3Ade was conducted in the same manner as on the simpler catechol structures using cesium carbonate as a base and DMF as a solvent. The estrogen-DNA adducts were synthesized in the same manner as reported previously [3]. These reactions produced highly fluorescent derivatives that could be detected at the low femtomolar level using reverse-phase HPLC separation coupled to a fluorescence detector. The derivatized estrogen-DNA adducts were also analyzed by fluorescence line narrowing spectroscopy (FLNS) using laser induced fluorescence excitation at low temperature. The FLNS spectra were collected at Iowa State University in collaboration with Dr. Ryszard Jankowiak. Dr. Jankowiak is trying to determine if the FLNS spectra can be used to distinguish the estrogen-DNA adduct derivatives from other anthracene containing metabolites or by-products. The FLNS spectra, coupled to capillary electrophoresis (CE) separation would provide a one-step analytical procedure to not only detect and quantitate the estrogen-DNA adducts from tissue samples, but also identify them by using the FLNS spectrum. Collaboration with Dr. Jankowiak continues in order to develop this analytical technique.

Work related to Task 3, "Isolate, quantitate and identify CE-adducts, CE and MPEM in breast tissue samples of pre- and post-menopausal women with and without breast cancer." This task was not completed due to a lack of access to breast tissue. However, extracted samples of breast tissue have been obtained from Dr. Jankowiak's laboratory. These samples are known to contain 4-OHE1-1-N3Ade estrogen-DNA adducts. The derivation of these samples, and conformation of the presence and identity of the 4-OHE1-1-N3Ade adducts is an ongoing collaboration with Dr. Jankowiak.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Extraction conditions for isolation of CE-DNA adducts, CE, and MPEM from rat breast tissue have been established.
- 2) Derivation procedure for fluorescence labeling of catechols has been developed using a three-step sequence of ketal formation, hydrolysis, and ester formation.
- 3) The synthesis of Bis-anthracen-9-ylmethyl-2,2-dibromo-malonamide has been conducted. This fluorescent probe, specific for the catechol moiety, allows fluorescent derivation of estrogen-DNA adducts in a one-step process. The resulting derivation products can be detected at low femtomolar levels using HPLC interfaced to a fluorescence detector.

REPORTABLE OUTCOMES:

- 1) A local grant entitled, "Establishment of Biomarkers for the Early Detection of Breast Cancer", was submitted and obtain from The University Council on Research, University of Nebraska at Omaha. The amount was \$8,300 and was used to match equipment funds needed for a new HPLC purchase.
- 2) Two Undergraduates, Clark Diffendaffer and Matthew Nammany, were employed full time during the summer of 1998. Their training in synthetic processes and analytical procedures not only provided for summer employment, but also furthered their academic goals relating to careers in health care.
- 3) Poster presentation, "Fluorescent Probes for Catechol Estrogens Using Esters Of α,α -Dibromomalonate", Era of Hope Meeting, Atlanta Georgia, June 9, 2000.
- 4) Poster entitled, "Synthesis Of Fluorescent Probes Specific For The Catechol Moiety" was presented at the annual national spring meeting of the American Chemical Society, San Diego, CA, April 1, 2001.
- 5) Nicole Burns, an undergraduate research assistant, presented a talk at the Nebraska Academy of Sciences annual meeting entitled, "Synthesis of Fluorescent Probes Specific for the Catechol Moiety", Lincoln, NE, April 27, 2001.
- 6) Two Undergraduates, Stacy Hill and Nicole Burns, were employed full time during the summer of 1999, part-time during the academic year 2000/2001, and full time during the summer of 2001. Nicole Burns received her Bachelors Degree in May 2001 and will continue her studies in breast cancer research at the University of Nebraska Medical Center as a graduate student.

CONCLUSIONS:

The development of an extraction procedure capable of isolating CE-DNA adducts, CE, and MPEM from rat breast tissue has been advanced. The extraction procedure is fast and amendable to the analysis of several samples using standard laboratory equipment. This procedure should translate to the isolation of estrogen metabolites from human tissue.

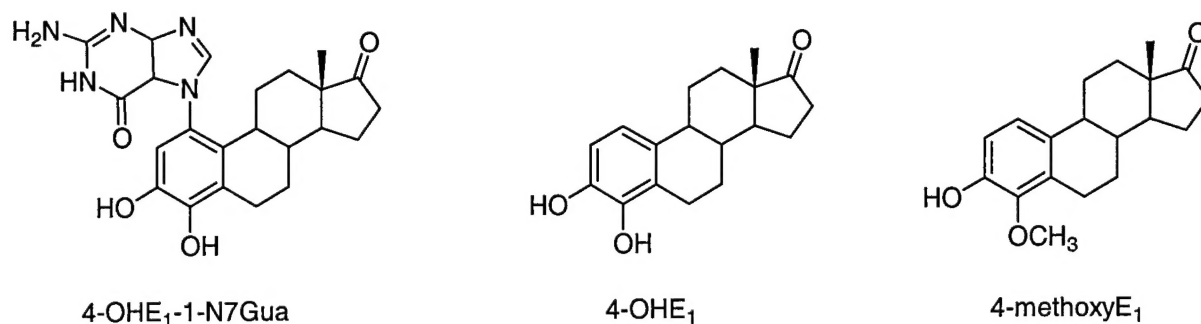
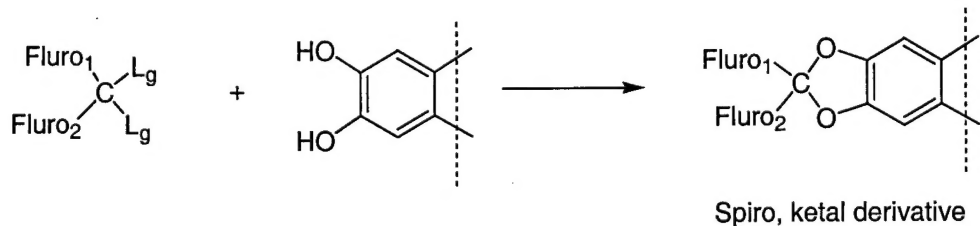
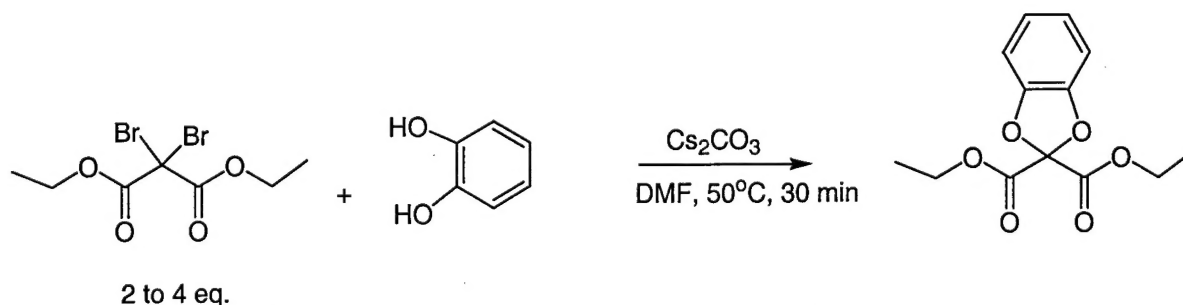
The development of a fluorescent probe for catechol estrogens (Task 2) has been completed. The best results were obtain by synthesis of N',N'-Bis-anthracen-9-ylmethyl-2,2-dibromo-malonamide. This probe contains very fluorescent anthracene moieties, in addition to a reactive *gem*-dihalide functional group that reacts specifically with catechols. The reaction between probe and catechol substrates occurs rapidly (0.5 h) at 50°C in the presence of cesium carbonate in a DMF solvent. The resulting derivative can be detected at the low femtomole level using HPLC separation interfaced to a fluorescence detector.

Task 3 was not completed; however, the derivation of pre-extract estrogen-DNA adducts from human breast tissue using the newly developed catechol probe is ongoing.

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APPENDIES:**Figure 1. Structures used to test extraction procedure.****Figure 2. Structural features of a successful fluorescent probe specific for the catechol moiety.****Figure 3. Reaction of diethyl α,α -dibromomalonate with catechol.**

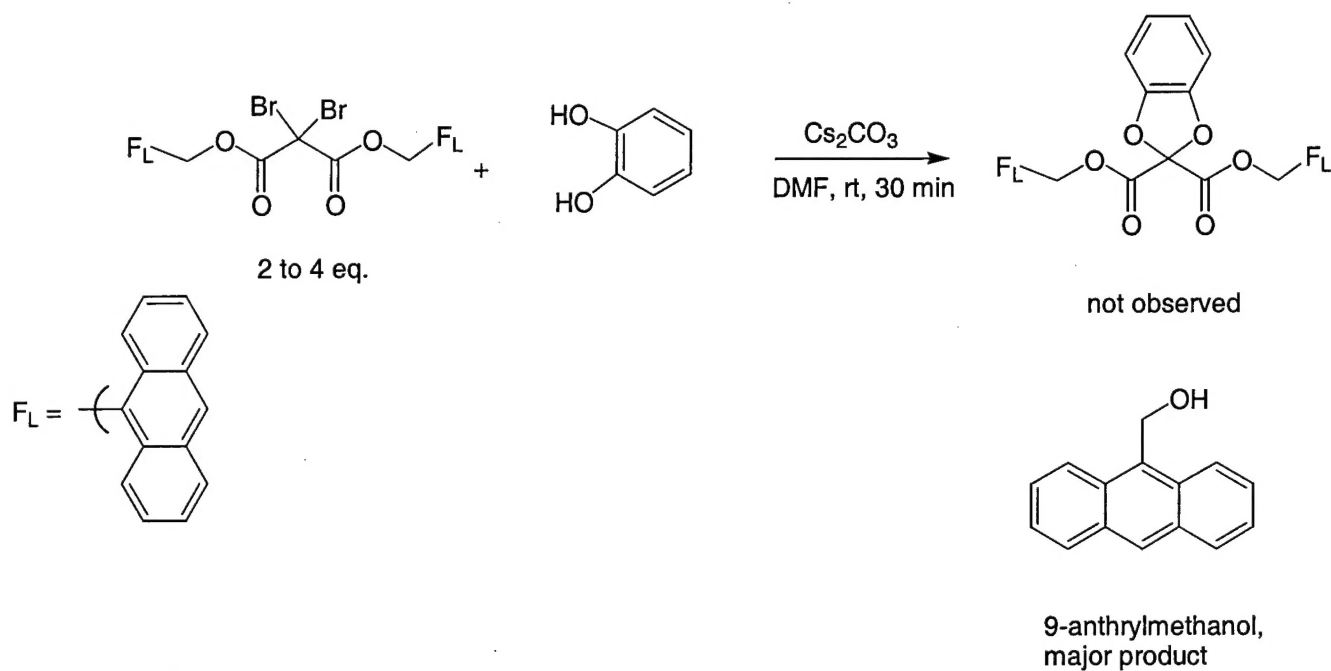


Figure 4. Reaction of catechol with bis-(9-methylanthyryl) α,α -dibromomalonate.

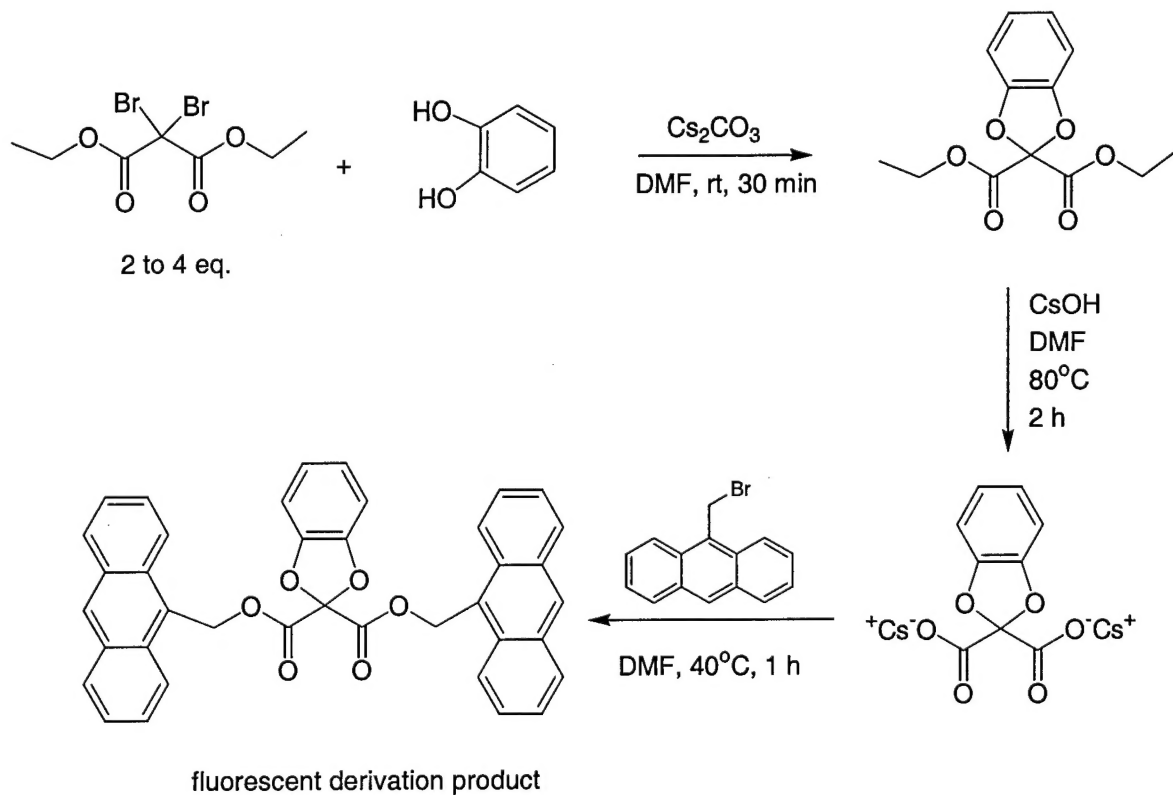


Figure 5. Three-step process for fluorescence labeling of catechols.

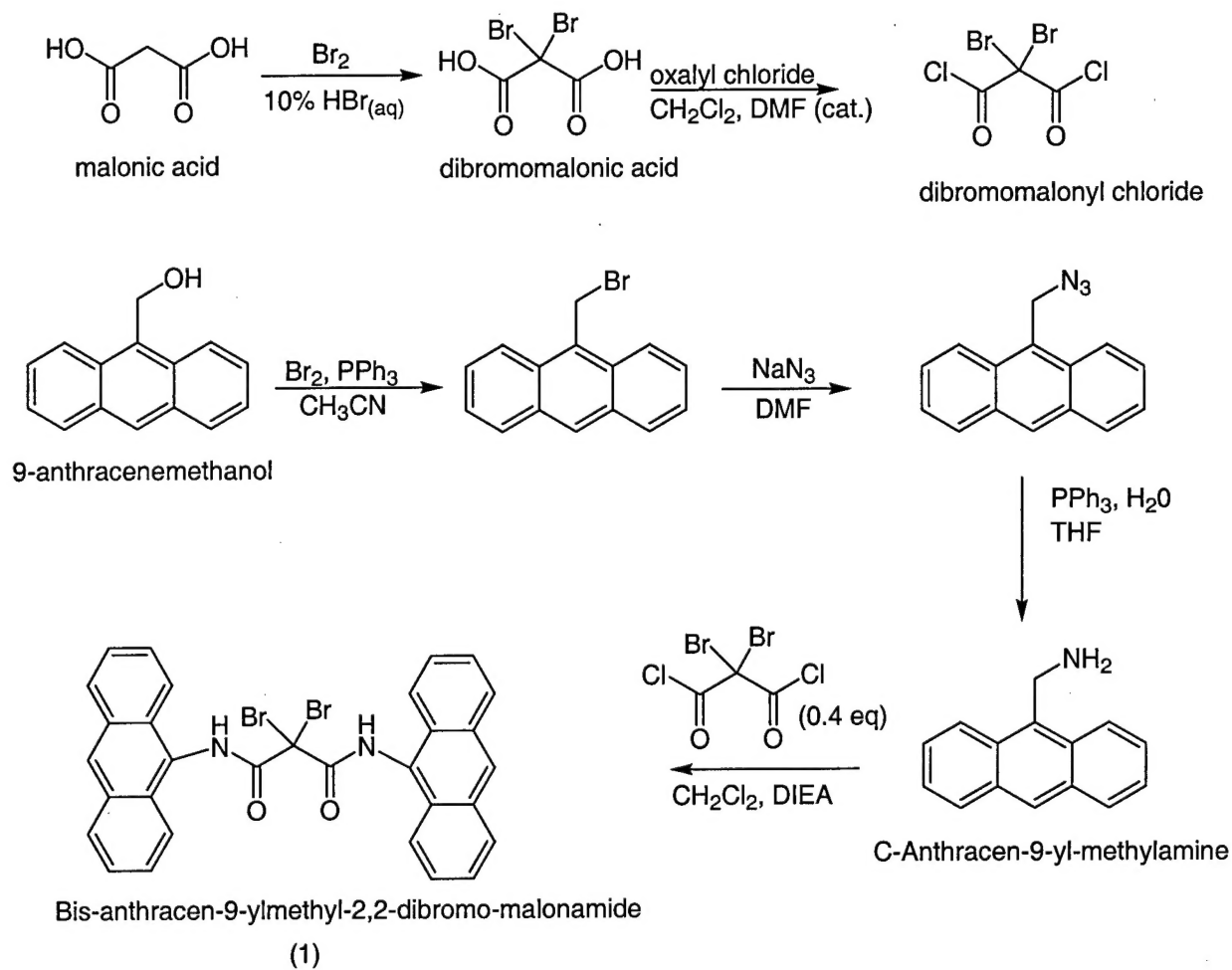


Figure 6. Synthesis of N,N'-Bis-anthracen-9-ylmethyl-2,2-dibromo-malonamide .

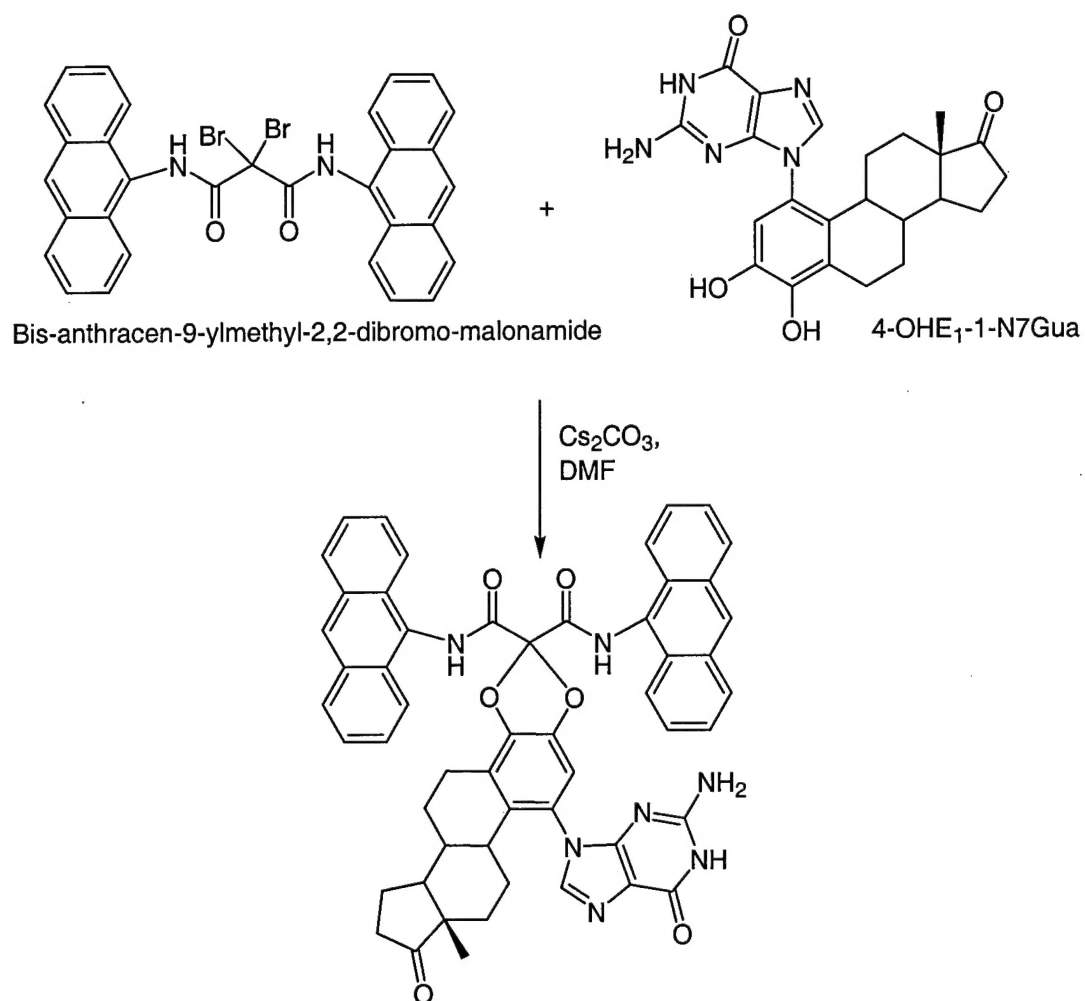


Figure 7. Reaction of N,N'-Bis-anthracen-9-ylmethyl-2,2-dibromo-malonamide with 4-OHE₁-1-N7Gua.

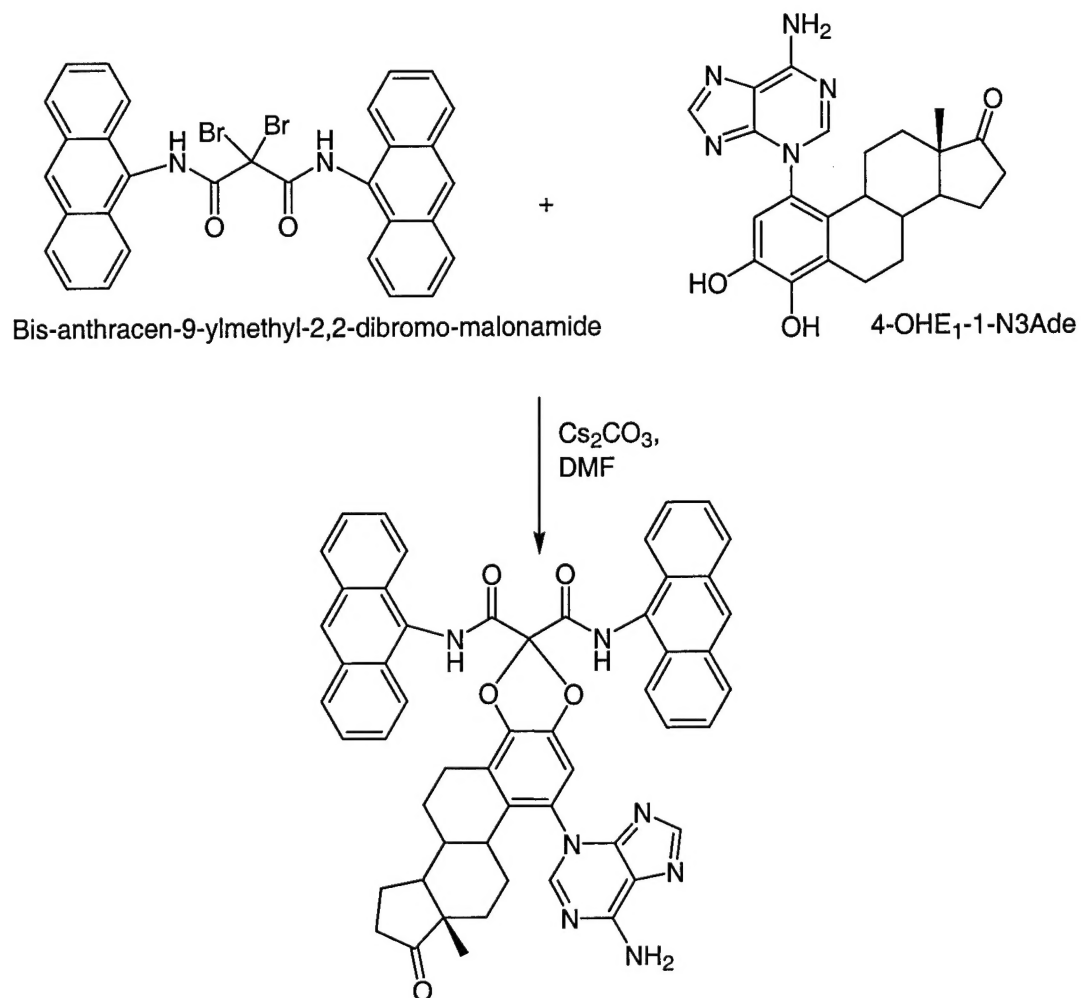


Figure 8. Reaction of N,N'-Bis-anthracen-9-ylmethyl-2,2-dibromo-malonamide with 4-OHE₁-1-N3Ade.